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Research Article

Barley Rhizosphere Bacteriome Dynamics under Organic and Mineral Inputs: The importance of intercropping predecessor

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Abstract

Integrating intercropping with green manure within crop rotation offers a sustainable pathway to enhance soil microbial diversity and overall agroecosystem health. This study evaluated the effects of diverse fertilisation practices on the soil microbiome in a barley (*Hordeum vulgare* L.) cropping system following the incorporation of an oat-vetch (*Avena sativa* and *Vicia sativa*) and green manure incorporation versus single cropping of oats. The field experiment took place at the Agricultural University of Plovdiv, Bulgaria. Using high-throughput metagenomic sequencing, we assessed the structure and functions of the soil microbial community. The fertilisation treatments included mineral fertiliser, vermicompost, a combination of vermicompost and mineral fertiliser, and biochar, along with a non-fertilised control. The investigation compared two preceding cropping systems before barley cultivation: an oat-vetch intercrop used as green manure, incorporated into the soil at the ripening stage, and a conventional approach where barley was sown directly without prior green manuring. All treatments were conducted in triplicate. Sequencing allows for detailed taxonomic and functional profiling. The most abundant bacterial phyla identified were Actinobacteriota, Proteobacteria, and Bacteroidota, with Actinobacteriota showing increased abundance under fertilised conditions. Soils treated with compost and compost-mineral mixtures exhibited notably higher alpha diversity. Beta diversity analyses (PCA, PCoA, UPGMA) revealed clear differences in microbial community structure among the treatments. Certain genera, such as *Sphingomonas*, *Noviherspirillum*, and *Agromyces*, were particularly enriched in soils receiving vermicompost or green manure. Their presence suggests enhanced microbial functionality and nutrient cycling in those plots. Overall, the combination of green manure and organic inputs supported more diverse and functionally active microbial communities, contributing to the resilience and sustainability of barley-based cropping systems. This study focuses on the bacterial component of the soil microbiome; nutrient cycling and soil health are governed by the combined interactions among bacteria, fungi, and protozoa.

Key words: Barley, high-throughput metagenomic sequencing, microbial diversity, plant-microbe interactions soil bacteriome, vermicompost

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Introduction

Microorganisms in the soil are key drivers of agroecosystem functionality, contributing to essential processes such as nutrient mineralisation, maintenance of soil structure, and promotion of plant development. The concept of the microbiome has evolved from a purely taxonomic description to a functional, systems-level understanding of microbial interactions in specific habitats (Berg et al. 2020). These microbial populations are involved in fundamental processes such as nutrient cycling, organic matter decomposition, and the regulation of plant health, directly influencing soil fertility and crop yields (Fierer et al. 2007; Van der Heijden et al. 2008). The structure and function of these microbial assemblages are closely linked to land management decisions, including fertilisation methods, cropping systems, and tillage intensity (Larkin 2015). As concerns about environmental sustainability grow, there is increasing attention on how agricultural interventions can maintain or improve microbial biodiversity, which is critical for the long-term productivity and stability of agroecosystems (Escudero-Martinez et al. 2022).

One strategy gaining traction is the integration of legumes into cropping systems through intercropping or green manuring. Legumes such as *Vicia sativa* L. can fix atmospheric nitrogen through symbiosis with rhizobia, enriching soil nutrient pools without the need for synthetic fertilisers (Peoples et al. 2009). The exudation of root metabolites from legumes can stimulate microbial activity and increase microbial richness in the rhizosphere. When incorporated into soil as green manure, legume biomass contributes organic inputs that enhance soil structure and biochemical function, creating a favourable environment for microbial growth and activity (Lazcano et al. 2013; Xiao et al. 2023). These effects often translate into improved performance of subsequent cereal crops, such as barley, through enhanced nutrient availability and disease suppression.

Organic inputs, including compost, vermicompost, and biochar, are also widely used to stimulate microbial processes and restore soil health. Compost and vermicompost provide easily accessible carbon and microbial inoculants that support enzymatic activity and microbial biomass development (Arancon et al. 2006; Lazcano et al. 2013). Biochar, a carbon-rich material derived from the pyrolysis of biomass, is valued for its porous structure and high surface area, which offer physical niches for microbial colonisation and enhance nutrient and water retention in soil (Lehmann and Joseph 2015). These amendments can contribute to building resilient soil microbial networks and improving overall soil functionality, particularly under organic or reduced-input systems (Lehmann et al. 2011).

Recent advances in sequencing technologies, including amplicon-based and metagenomic approaches, now enable researchers to dissect microbial community dynamics at high resolution. By targeting genes such as the 16S rRNA or applying shotgun metagenomics, scientists can simultaneously assess taxonomic diversity and the metabolic potential of soil microbial communities (Nissimov 2017; Prosser 2020). Despite these developments, field-based studies that integrate legume intercropping, organic amendments, and microbial community profiling remain limited, especially under temperate European conditions. This study investigates the impact of incorporating a legume-cereal mixture (*Avena sativa* × *Vicia sativa*) as green manure, followed by spring barley cultivation, on the composition and function of rhizosphere bacterial communities under varying fertilisation regimes. Microbial community structure and functional potential

were assessed using 16S rRNA gene sequencing and metagenomic analysis. The present study aims to reveal how fertiliser type and the legacy effects of cropping system influence soil bacteriome diversity in the rhizosphere of fodder winter barley. This study focuses on the bacterial component of the soil microbiome and recognises that nutrient cycling and soil health are shaped by the combined activities of bacteria, fungi, and protozoa within the broader soil food web.

Materials and methods

Field description and climatic conditions

The field trial was carried out in 2023 at the Training and Experimental Station of the Agricultural University of Plovdiv, Bulgaria (42°08'15.4"N, 24°48'16.4"E). The soil at the site is classified as Mollic Fluvisol, according to the FAO system, formed on alluvial deposits with a humus-rich upper horizon and a transition into a carbonate layer. The region falls within the temperate continental climate zone of Southern Bulgaria, suitable for long-term agroecological experimentation (Shilev et al. 2024). The environmental conditions during the study could be characterized as average, taking into account the average values of multi-year data. The temperatures did not show significant variations from the optimal for the barley development. The average amount of precipitation at the end of 2022 was 75 mm, while at the beginning of 2023, it was 138 mm, with a lower amount in March.

Experimental design and soil amendments

The study was structured within a two-year crop rotation scheme, aimed at assessing the legacy effects of legume-based green manure and different fertilisation practices on soil microbial diversity. In spring of the first year (2022), two different cropping strategies were implemented using a randomized block layout with three replicates per treatment: (i) monoculture oats (*Avena sativa* L., cv. Max), and (ii) intercropped oats with vetch (*Vicia sativa* L., cv. Obrazets 666) at a 3:1 seeding ratio (vetch: oats), totaling 220 kg/ha. Each plot measured 5 × 7 m (35 m^2). Sowing was done in March 2022. The monocrop oats were harvested at the end of June, while the intercropped biomass was ploughed into the soil as green manure at a depth of 30 cm. In the same year (2022), at beginning of November, all plots were cultivated with winter barley (Zemela cultivar) to evaluate residual and interactive effects of prior cropping and fertilisation schemes. Two main field setups were established: (1) plots previously enriched with green manure, and (2) conventional plots without green manure. Five fertilisation treatments were applied to both fields: no fertilisation (Control1, Control2); mineral fertiliser (MF1, MF2), NPK 15:15:15, 100 kg/ha, vermicompost (VC1, VC2), 12 t/ha; combined mineral fertiliser and vermicompost (MF1.VC1, MF2.VC2) at half concentrations; and biochar (Bch1, Bch2), 10 t/ha. In this study, different organic amendments were applied and are defined as follows: Compost (Com) refers to mature plant-based compost applied alone; Vermicompost (VC) is compost processed through the activity of earthworms; and Biochar compost (BC or BC+Com) denotes a mixture of biochar blended with compost prior to soil application. For clarity, the term organic inputs is used throughout the manuscript as

a general descriptor for all organic treatments (Com, VC, and BC+Com), whereas individual abbreviations are employed when specific treatments are discussed. All treatments were arranged in a randomised complete block design with three replicates per treatment, resulting in a total of 30 barley plots. At the ripening stage of barley, rhizosphere soil samples were collected from each plot by randomly selected plants. This layout enabled the study of fertilisation and green manuring effects on rhizosphere microbial structure under field conditions (Shilev et al. 2024). Samples were kept on ice during transport, sieved to 2 mm, and stored at −80 °C until DNA extraction (Kulmatiski 2020; Natalio et al. 2024). DNA was extracted from 0.5 g of soil using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following standard protocols.

The analytic data showed sufficient concentrations of most macroelements as total content in the biochar (nitrogen, potassium, calcium, and magnesium) (Table 1). The phosphorus and iron content were higher in the vermicompost. The analytical procedure is described previously by Shilev et al. 2024.

The soil analyses at the beginning of the study have shown a relatively low total organic carbon content (TOC) – 1.14% (Table 2). Soil reaction was slightly alkaline with low electrical conductivity (EC). The soil is poorly supplied with nitrogen and well stocked with phosphorus and potassium.

Metagenomic sequencing

DNA quality control

Extracted DNA quality and quantity were assessed using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and 1% agarose gel electrophoresis. All samples met the quality standards defined by Novogene Co. Ltd., including parameters for DNA integrity, purity, and concentration (Schloss et al. 2009).

Table 1. Properties of vermicompost and biochar on a dry weight basis. Data show mean and standard error (n = 3).

	Unit	Vermicompost	Biochar
TOC	(%)	18.08 ± 1.48	65.00
pH		7.79 ± 0.01	–
EC	($\mu S\ cm^{-1}$)	1685 ± 10.0	–
Total N	(%)	1.29 ± 0.17	1.31
P	(%)	1.05 ± 0.08	0.64
K	(%)	1.61 ± 0.21	7.95
Ca	(%)	3.11 ± 0.32	4.28
Mg	(%)	0.72 ± 0.07	3.06
Fe	(%)	0.51 ± 0.05	0.13

Table 2. Physicochemical properties of the soil before application of amendments on a dry weight basis. Data show mean and standard error (n = 3).

	pH	EC ($\mu S\ cm^{-1}$)	Accessible N ($N\text{-}NH_4$+$N\text{-}NO_3$) ($mg\ 100\ g^{-1}$)	P_2O_5 ($mg\ 100\ g^{-1}$)	K_2O ($mg\ 100\ g^{-1}$)	TOC (%)
Soil	8.22 ± 0.1	121.0 ± 1.7	1.54 ± 0.03	3.58 ± 0.28	9.9 ± 0.1	1.14 ± 0.09

Library preparation and sequencing

Metagenomic libraries were constructed through DNA fragmentation, end-repair, A-tailing, and adapter ligation, following Illumina's standard protocol. Where necessary, libraries underwent PCR amplification. Following quantification and quality control, sequencing libraries were diluted to 1.5 nM and subjected to paired-end sequencing (2 × 150 bp) on the Illumina NovaSeq 6000 system (Quince et al. 2017).

Bioinformatic analysis

Data preprocessing and quality control

The raw sequencing data underwent quality control with fastp v0.23.1 (Chen et al. 2018a), which involved removing adapter sequences, reads containing ambiguous nucleotides, and those with quality scores below Q20. Barcode and primer regions were trimmed before merging the high-quality paired-end reads using FLASH v1.2.11 (Magoč and Salzberg 2011). Detection and removal of chimeric sequences were performed using vsearch v2.16.0 (Rognes et al. 2016), referencing the SILVA v138 database (Quast et al. 2013) for improved accuracy.

Feature table construction and taxonomic assignment

Amplicon Sequence Variants (ASVs) were inferred using the DADA2 plugin in QIIME2 v2022.2 (Callahan et al. 2016). Taxonomic classification was conducted using a Naïve Bayes classifier trained on the 515F/806R region from the SILVA v138 reference set. ASV tables were rarefied to the minimum read depth for downstream comparisons.

Diversity and community composition

Alpha diversity indices (Chao1, Shannon, Simpson, Pielou's evenness, Good's coverage) and beta diversity metrics (Bray-Curtis, weighted and unweighted UniFrac) were calculated using QIIME2. Ordination analyses including Principal Coordinates Analysis (PCoA), Principal Component Analysis (PCA), and Non-metric Multidimensional Scaling (NMDS) were conducted in R v4.2.0 (R Core Team 2022) using the vegan, ggplot2, and ade4 packages. Group-level differences were assessed via Analysis of Similarities (ANOSIM) and Permutational Multivariate Analysis of Variance (PERMANOVA) using the vegan package (Anderson 2001)

Phylogenetic and statistical analyses

Multiple sequence alignments of representative ASVs were performed with MAFFT (Katoh and Standley 2013), and phylogenetic trees were constructed using FastTree in QIIME2 (Price et al. 2010). Visualisation of microbial composition was carried out using stacked bar plots, heatmaps (heatmap), and ternary plots, implemented in R software v4.2.0 (R Core Team 2022; https://www.r-project.org/ accessed on 06.07.2025). Venn and flower diagrams representing shared and unique ASVs were generated using the VennDiagram package and custom SVG-based scripts.

Data availability

The 16S rRNA sequencing data from examined soils were submitted to the NCBI gene bank under the BioProject accession number PRJNA1297989 with the submission ID: SUB15499394 released on 29 July 2025. The BioSample accessions of the analysed ten samples are SAMN50254222, SAMN50254223, SAMN50254224, SAMN50254225, SAMN50254226, SAMN50254227, SAMN50254228, SAMN50254229, SAMN50254230, and SAMN50254231. The data records are accessible using the following link: https://www.ncbi.nlm.nih.gov/bioproject/1297989, accessed on 29 July 2025.

Results

Sequencing data processing

Row PE (Paired-End Reads) values range from 62,941 (MF2) to 78,820 (VC1), which is a reasonable range for 16S rRNA gene amplicon sequencing (Table 3). No sample shows an extreme outlier, meaning sequencing depth was fairly consistent. The combined (Merged Reads) step merges the paired-end reads into longer sequences. The merging rates are high across all samples (close to 100%), indicating good sequencing quality and proper overlapping read regions. Successful merging suggests accurate read alignment without major issues in adapter trimming or sequence quality. Qualified (Clean Tags in Table 3). The percentages remain high across all samples, showing data underwent minimal loss due to low-quality reads. This reflects that sequencing run had high quality, and data loss at this stage was minimal. Nochime (Effective Tags) indicates the presence of chimaeras. Control1 dropped from 63,766 to 54,656 (~14.3% reduction), and VC1 dropped from 77,093 to 70,624 (~8.4% reduction). Chimaera rates vary slightly between samples. MF1.VC1 and Bch2 show lower chimaera rates, indicating these samples had fewer artefacts. GC content ranges from 56.03% to 57.02%, which is biologically consistent and within the expected range for most microbial communities. All samples exhibit Q20 > 98% and Q30 > 95%, demonstrating excellent sequencing accuracy. The high Q20/Q30 scores confirm minimal sequencing errors and high data reliability.

Analysis of relative taxonomic abundance

This stacked bar plot represents the relative abundance of bacterial taxa at the phylum level across different samples (Fig. 1). The bar plot of relative abundance at the phylum level illustrates consistent dominance of Proteobacteria, Actinobacteriota, and Acidobacteriota across all treatments, in line with their established roles in nutrient cycling and soil health. The proportional distribution of these and other phyla varied depending on the fertilisation regime and the application of green manure.

Treatments VC, MF.VC, and to a lesser extent Bch, showed elevated levels of Actinobacteriota and Bacteroidota, phyla known for their saprophytic and degradative capabilities. This shift suggests increased availability of organic substrates following the incorporation of legume biomass, which can stimulate microbial taxa involved in organic matter decomposition and nutrient release.

Table 3. Sequencing data analysis.

Sample	RawPE	Combined	Qualified	Nochime	Base(nt)	Avglen(nt)	GC, %	Q20,%	Q30,%
Control1	65518	64580	63766	54656	22898990	418.97	56.65	98.71	95.43
MF1	66110	65551	64810	59174	24743668	418.15	56.10	98.87	95.80
VC1	78820	78024	77093	70624	29567160	418.66	56.03	98.86	95.81
MF1.VC1	68808	68308	67339	59460	24800258	417.09	56.27	98.74	95.35
Bch1	66344	65837	64947	56883	23728533	417.15	57.02	98.77	95.48
Control2	70347	69768	68827	64043	26761127	417.86	56.74	98.81	95.70
MF2	62941	62431	61527	55209	23012585	416.83	56.74	98.70	95.21
VC2	70455	69639	68724	62112	25927482	417.43	56.97	98.75	95.58
MF2.VC2	63834	63011	62111	54694	22854201	417.86	56.47	98.74	95.42
Bch2	64928	64102	63302	54227	22642699	417.55	56.51	98.75	95.54

Legend – Abbreviations of sample codes: Control1-unfertilized control after green manure, Control2 – Barley grown without fertilisation ; MF1-Barley grown after oat-vetch green manure and fertilized with mineral fertilizer, MF2 – fertilized with mineral fertilizer, VC1 - Barley grown after oat-vetch green manure and fertilized with vermicompost, VC2 –fertilized with vermicompost, MF1.VC1 – Barley grown after oat-vetch green manure, fertilized with compost + mineral fertilizer, MF2.VC2 – fertilized with compost + mineral fertilizer, Bch1 - Barley grown after oat-vetch green manure, fertilized with biochar, Bch2 – fertilized with biochar only.

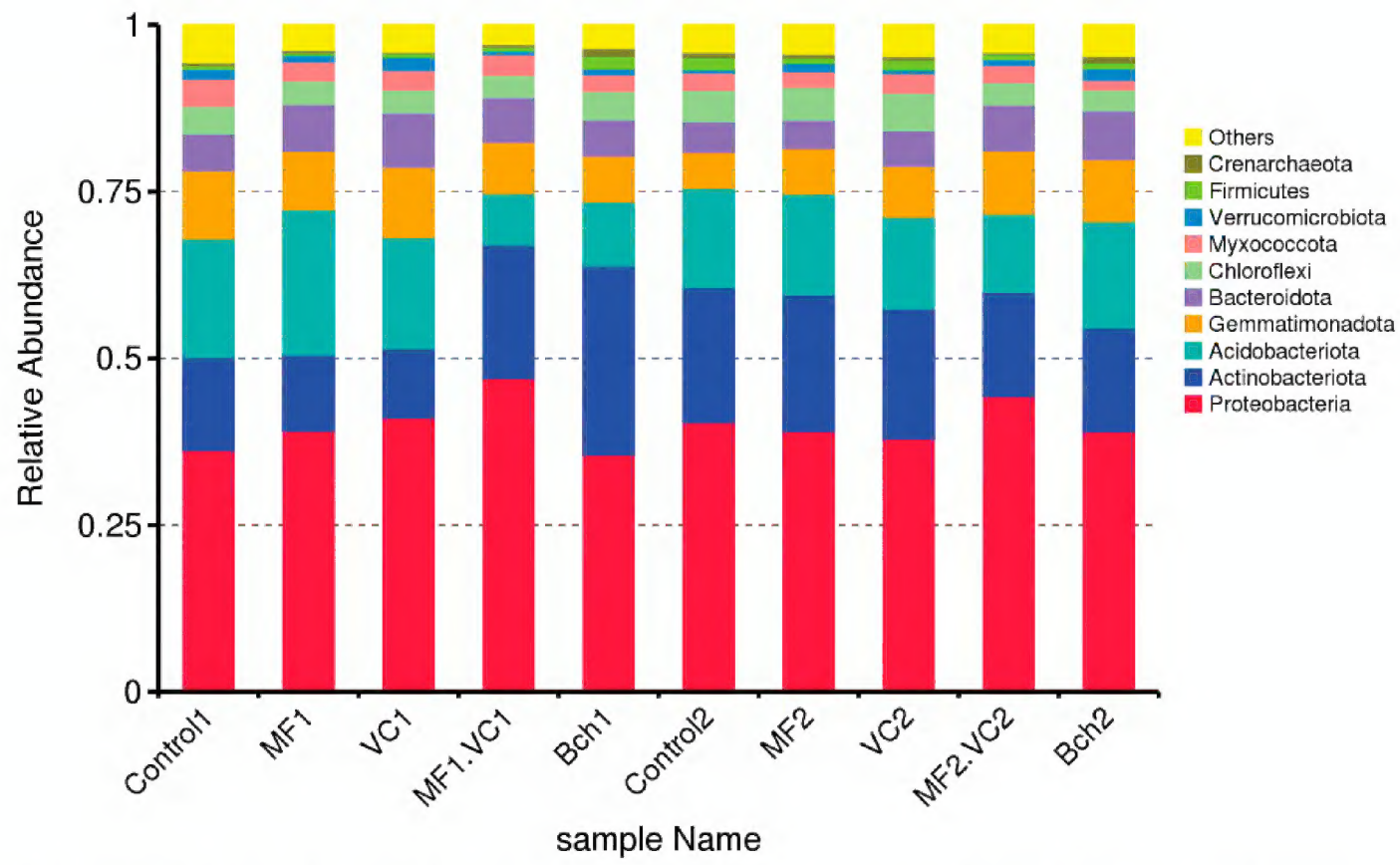

Figure 1. Relative taxonomy abundance of each sample in different taxonomic level. at phylum level.

In contrast, plots treated with mineral fertilizer or left as untreated controls exhibited relatively higher proportions of Acidobacteriota, often associated with oligotrophic conditions and more stable, slower nutrient cycling dynamics (Fig. 1). These treatments also showed reduced abundance of phyla such as Gemmatimonadota and Myxococcota, indicating a less diverse and less functionally dynamic microbial community. The biochar treatment presented a unique phylum distribution, with slightly elevated Verrucomicrobiota and Chloroflexi, consistent with previous findings that biochar alters microhabitat conditions such as aeration, pH, and moisture content, thereby selecting for specialized microbial taxa. The results underscore that green manure incorporation, particularly in combination with compost or mineral fertilizers, enhances microbial diversity and shifts the taxonomic structure toward copiotrophic, functionally active taxa. These microbial changes reflect improved soil fertility and biological functioning under intercropping-based, organic-amended systems.

Analysis of relative taxonomic abundance at the genus level

The genus-level bar plot (Fig. 2) reveals a consistent dominance of *Sphingomonas* across all treatments, highlighting its ubiquity and potential ecological relevance in the barley rhizosphere. This genus, known for its capacity to degrade complex organic compounds and promote plant health, was most abundant in compost- and biochar-amended soils, particularly in MF.VC and VC treatments. This suggests that green manure incorporation enriched copiotrophic genera linked to nutrient transformation and organic matter decomposition.

Other notable genera include *Lysobacter*, *Noviherspirillum*, and *Gemmatimonas*, which together contributed to a moderate proportion of the community in Fig. 2. These genera are often associated with carbon and nitrogen cycling and showed slight increases in compost and biochar treatments, possibly reflecting the enhanced availability of root exudates and organic substrates. In contrast, control and mineral-only (MF) treatments exhibited lower richness and diversity at the genus level, with greater proportions classified under "Others," indicating either lower taxonomic resolution or dominance of rare/uncultured taxa. *Massilia* and *Arenimonas* were more prominent in biochar-amended soils (Bch1, Bch2), suggesting their potential preference for the altered physicochemical properties (e.g., pH, porosity) associated with biochar. These results confirm that green manure, particularly when combined with organic amendments, enriches beneficial microbial taxa at the genus level, contributing to improved soil health and potential plant growth-promotion under intercropping systems.

Heat map of genus levels

The heatmap highlights the differential abundance of dominant bacterial genera across treatments, clustered both by taxonomic affiliation and treatment type (Fig. 3). Samples treated with green manure, especially MF.VC, and VC, exhibited clear shifts in microbial composition compared to mineral fertilisation (MF) and untreated controls. Genera such as *Nocardioides*, *Agromyces*, *Rubrobacter*, *and Solirubrobacter* (phylum: Actinobacteriota) were notably

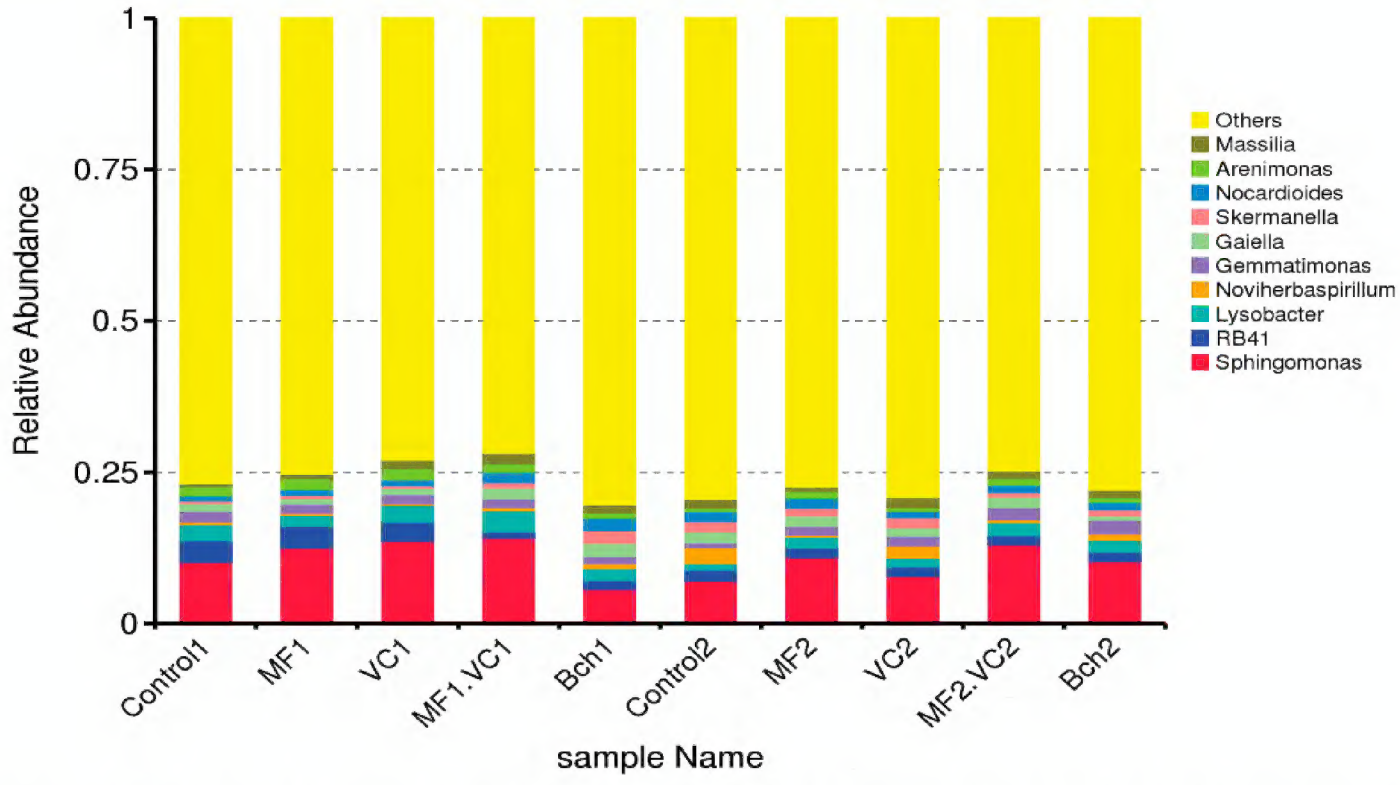

Figure 2. Relative taxonomy abundance of each sample in different taxonomic level at genus level.

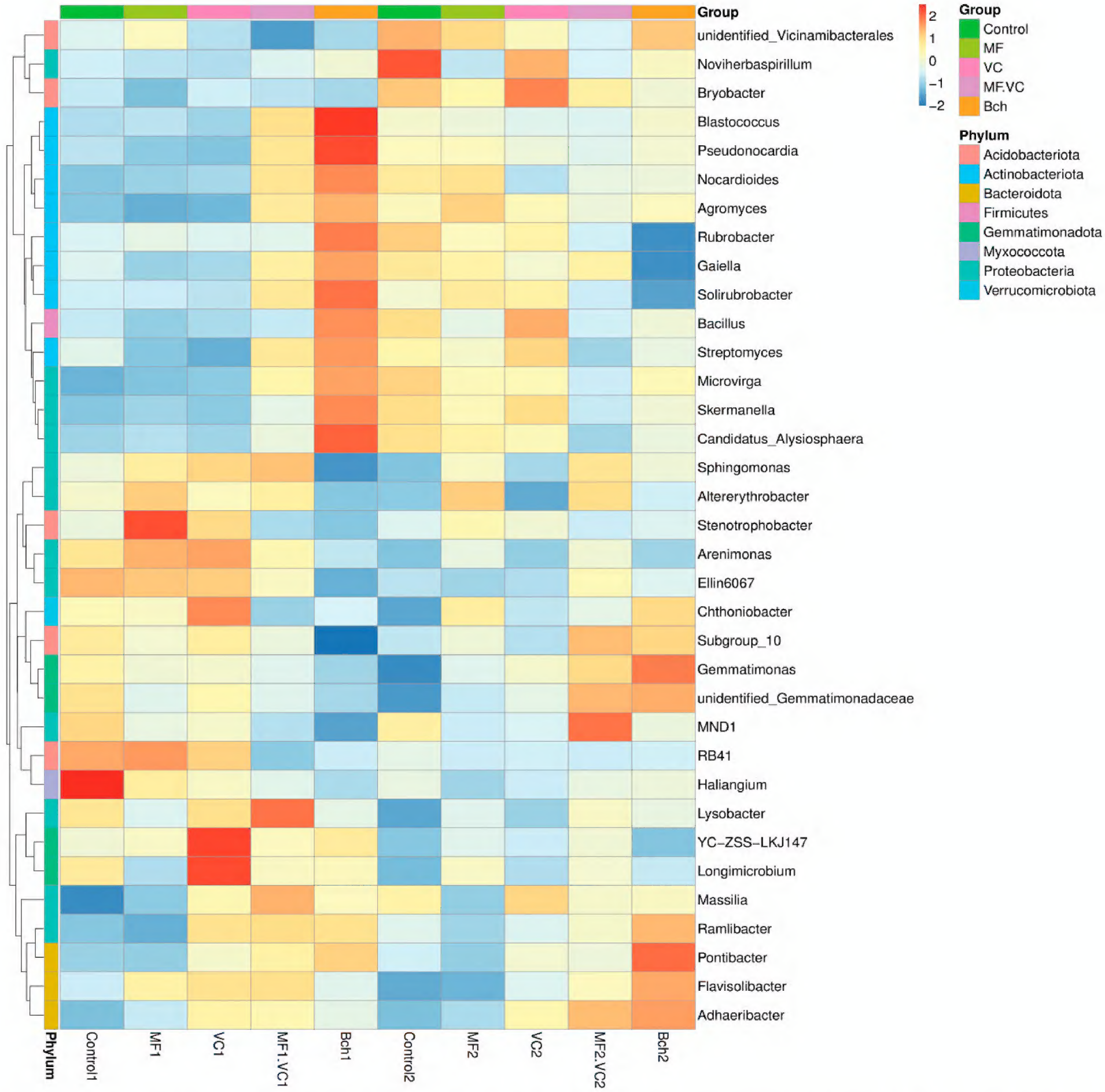

Figure 3. Heatmap showing the relative abundance of the 35 dominant genera across all samples. The X-axis represents sample codes, and the Y-axis represents genera. Hierarchical clustering is shown on the left. The Z-score indicates deviation from the mean abundance, and the colour gradient reflects abundance levels: red/orange = higher, yellow = intermediate, blue = lower.

enriched in green manure and biochar (Bch) treatments, reflecting increased microbial activity related to organic matter decomposition. These genera are often associated with cellulose degradation, plant-growth promotion, and resilience to environmental stress.

Conversely, *RB41*, *Lysobacter*, *and Sphingomonas* were more abundant in control and mineral fertilizer treatments. Their prevalence in nutrient-limited environments may indicate a competitive advantage in lower-carbon soils. The relatively lower presence of copiotrophic genera in MF and Control samples suggests reduced microbial richness and functional diversity. Distinct patterns were also evident for *Noviherspirillum* (Proteobacteria), which showed

treatment-specific enrichment in MF.VC, consistent with its role in nitrogen cycling and plant-microbe symbiosis. Similarly, *Blastococcus and Bryobacter* were highly enriched in VC-treated plots, suggesting stimulation of oligotrophic microbes by compost-derived substrates. The clustering of samples by microbial profiles further supports the conclusion that green manure and compost-based treatments significantly alter microbial community structure, promoting taxa with specialized ecological functions. This community restructuring likely contributes to improved soil health and nutrient cycling efficiency in intercropped systems.

Bacteriome abundance at species level

Several species exhibit high abundance in specific groups. *Acidobacteria bacterium* shows notable peaks in MF1.VC1 and MF2.VC2, suggesting an association with mineral fertilisation (Fig. 4). *Steroidobacter sp*. and *Pontibacter*_sp_LX8 are enriched in Control2 and MF2, indicating their potential role in stable environments. *Adhaeribacter terrae* and *Lysobacter fragariae* are present at higher levels in MF1 and MF1.VC1, potentially linked to treatment conditions with mineral additive.

Control soil samples in Fig. 4 (Control1, Control2) generally show balanced microbial profiles with moderate abundance variations, reflecting stable microbial communities. MF1 and MF2 exhibit clear enrichment of specific species like *Acidobacteria bacterium* and *Pontibacter*, which may indicate microbial responses to experimental conditions. VC samples present a mix of moderate and low-abundance species with less distinct clustering patterns. Soils with Bch1 and Bch2 demonstrate some unique peaks in species like *Caenimonas* and *Lysobacter* soil, indicating distinct microbial adaptations.

Thus, control samples display balanced community profiles, indicating microbial stability. MF and VC samples show distinct microbial shifts, suggesting an effect of experimental treatments. The presence of unique peaks in species like *Caenimonas*, *Rhodoferax*, and *Aquimonas* may highlight microbial adaptation to specific environmental conditions. Stable dominance of Actinobacteria and Acidobacteriota indicates that they are core microbiome members in these environments.

Microbial community composition across soil treatments

The flower Venn diagram illustrates in Fig. 5 the distribution of unique and shared microbial taxa identified through 16S rRNA gene amplicon sequencing across the different soil treatments. A total of 363 microbial taxa formed the core microbiome, being consistently present in all treatments, regardless of fertilisation regime. This core set likely represents functionally important and environmentally resilient taxa that maintain essential soil processes. The highest numbers of treatment-specific taxa were detected in the compost-amended plots, particularly VC2 (837 taxa) and VC1 (779 taxa), which received green manure derived from an oat-vetch incorporated into the soil during the first year of the experiment (Petkova et al. 2025). The combination treatments (MF1.VC1 with 558 unique taxa and MF2.VC2 with 527 taxa) also demonstrated enhanced microbial richness, suggesting a synergistic effect between mineral fertilizers

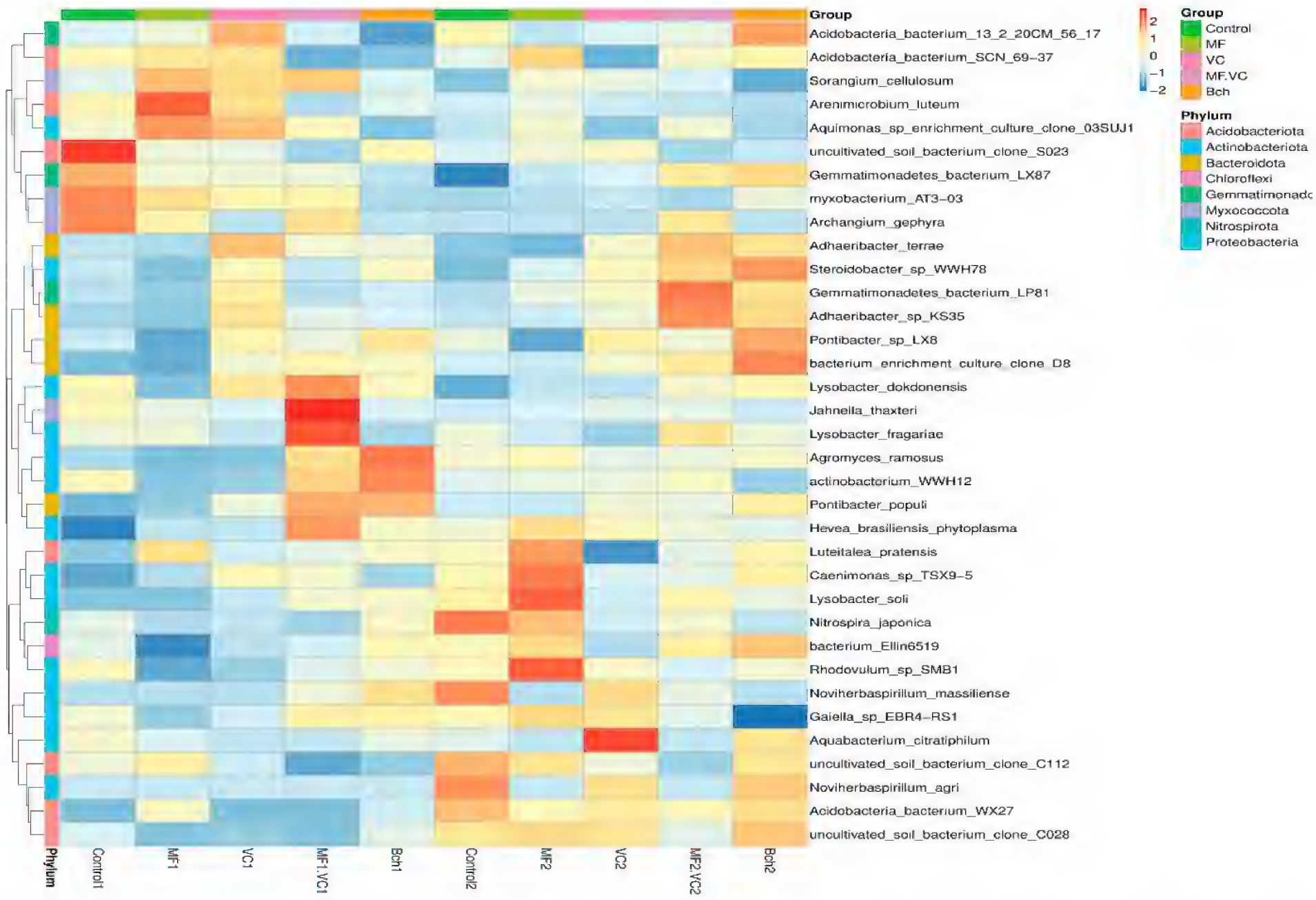

Figure 4. Heatmap visualizes the relative abundance of dominant species across different sample groups, with colors representing abundance intensity: Red/Orange = Higher abundance, Blue = Lower abundance, Yellow/White = Intermediate abundance.

and organic amendments. Bch1 and Bch2 contributed distinct microbial communities with 657 and 532 unique taxa, respectively, reflecting the biochar's known capacity to modify soil physicochemical properties and influence microbiome assembly. Control1 and Control2 retained a relatively high number of unique taxa (676 and 703, respectively), indicating a rich native microbial diversity in the untreated soil (Fig. 5). Collectively, these results underscore the substantial impact of fertilisation strategy—particularly the use of legume-based green manure and its integration with mineral inputs—on the diversity and specificity of soil microbial communities. The observed shifts suggest that agroecological practices involving compost and intercropping may enhance microbial functional potential and contribute to sustainable soil fertility.

The evolutionary tree in genus level

To further study the phylogenetic relationship of the genus, the top 100 genera were selected and the evolutionary tree was drawn using the aligned representative sequences (Fig. 6). Proteobacteria dominates a significant portion of the tree, reflecting its common presence in diverse ecosystems. Actinobacteriota and Bacteroidota are also highly represented, aligning with their known abundance in soil and environmental samples. Firmicutes and Verrucomicrobiota appear less frequently but are distinctly clustered. Chloroflexi, Acidobacteriota, and Gemmatimonadota are present but in smaller proportions.

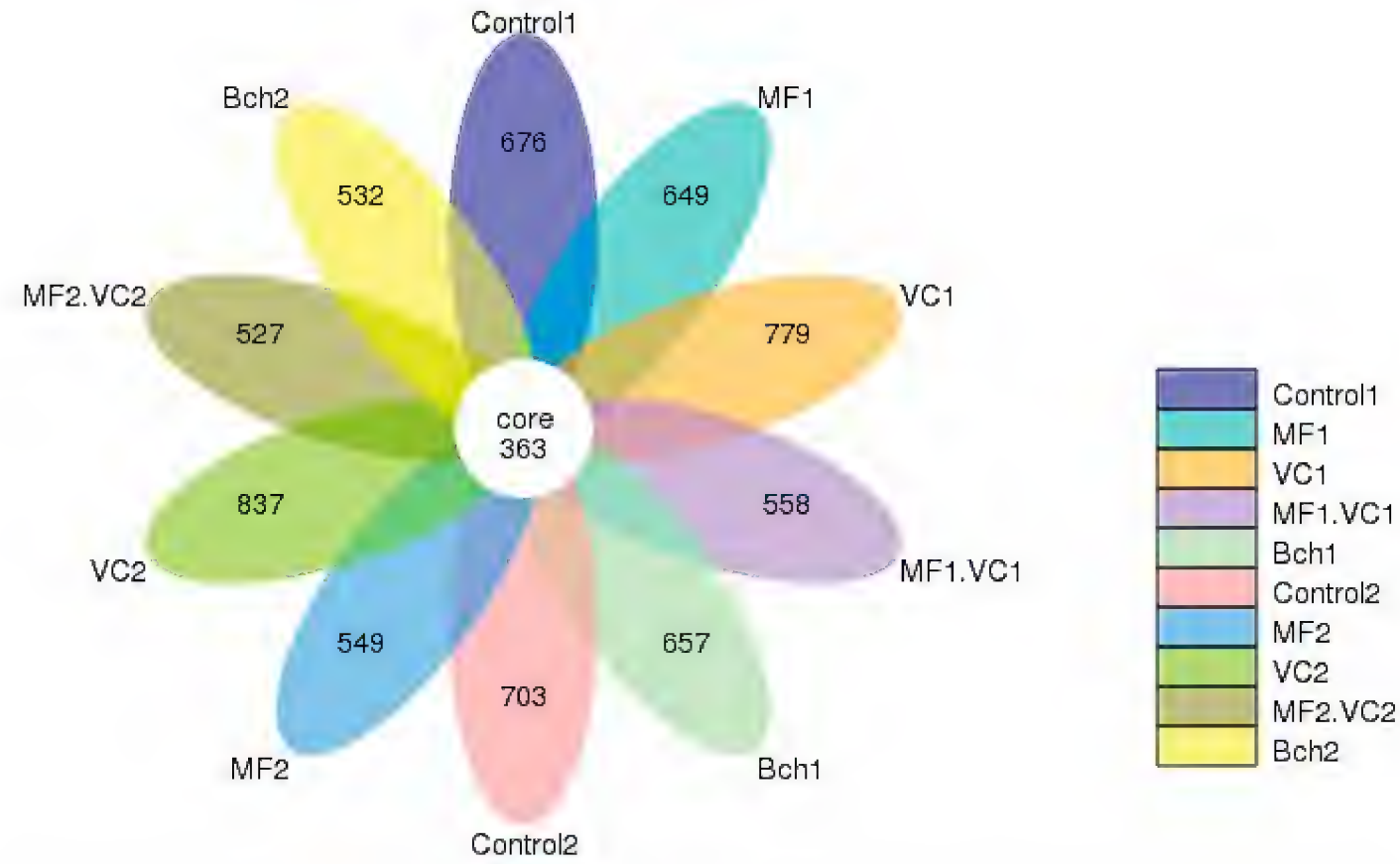

Figure 5. The Venn diagram illustrates the shared and unique species across five different sample groups of each field.

Control1, MF1, and VC1 display widespread microbial presence across multiple genera (Fig. 6). Distinct enrichment patterns are noticeable in Bch1 and Bch2 — showing dominance in select Actinobacteriota and Acidobacteriota. MF1.VC1 and MF2.VC2 displaying notable peaks in certain Proteobacteria and Bacteroidota. The presence of rare phyla like Crenarchaeota and Entotheonellota may indicate unique microbial niches in specialized environments with the addition of mineral fertilizers. Proteobacteria, Actinobacteriota, and Bacteroidota dominate the microbial community, reflecting their ecological resilience. Closely related genera cluster together, particularly within phyla such as Proteobacteria, Bacteroidota, and Actinobacteriota. Genera within the *Myxococcota* and *Nitrospirota* phyla cluster tightly, indicating closely conserved evolutionary traits. The distinct evolutionary branches of *Crenarchaeota* and *Entotheonellota* suggest their unique ecological roles and evolutionary paths. Proteobacteria dominance reflects its versatility in metabolizing diverse substrates, making it essential in nutrient cycling and environmental resilience. The presence of Actinobacteriota and Firmicutes suggests potential antibiotic-producing species or those involved in organic matter decomposition. Enriched Verrucomicrobiota and Nitrospirota may indicate species with specialized metabolic pathways suited for specific environmental conditions.

Bacteriome diversity indices

Alpha diversity analysis

Alpha diversity measures the richness and diversity within a single sample (Li et al. 2013). It is illustrated through species accumulation plots, biodiversity curves, and related statistical analyses (Table 4). Alpha diversity metrics assess the richness, evenness, and overall diversity within individual samples. In the dataset, key metrics like Chao1, Observed Features, Shannon, and Pielou's evenness provide valuable insights. VC2 (Chao1: 2868.429) and VC1 (Chao1:

Figure 6. The circular phylogenetic tree visualizes the evolutionary relationships between the top 100 genera identified in soil samples. It integrates taxonomy, sample abundance, and microbial diversity.

2864.731) exhibit the highest species richness. Bch2 (Chao1: 2315.000) and MF2.VC2 (Chao1: 2379.850) have the lowest richness values. Control samples (Control1 and Control2) show intermediate richness values, indicating a diverse but slightly less rich community than VC groups. VC1 and VC2 appear to host the richest microbial communities, suggesting higher environmental complexity or better conditions for microbial proliferation. The reduced richness in Bch2 and MF2.VC2 may indicate environmental stress or selective pressure.

Pielou's Evenness (E) measures species distribution uniformity (Table 4). VC2 (Pielou's E = 0.921) and Bch1 (Pielou's E = 0.920) exhibit the highest evenness, reflecting balanced community structures. MF1.VC1 (Pielou's E = 0.899) has the lowest evenness, suggesting a more dominant species or reduced community balance. Evenness is generally high across all samples, indicating a well-distributed microbial population in most conditions. Diversity indices like Shannon and Simpson have been analysed to calculate the richness and evenness, where the higher values indicate greater diversity. VC2 (Shannon = 10.572) and Control2 (Shannon = 10.443) have the highest diversity. VC2 and Control2 show the most diverse and stable microbial ecosystems. MF1.VC1 (Shannon = 10.095) and MF2.VC2 (Shannon = 10.175) show slightly lower diversity scores. Simpson Index is uniformly high (~0.998–0.999) across all samples, suggesting consistently diverse ecosystems. MF1.VC1 and MF2.VC2 display slightly reduced diversity, which may reflect environmental pressures or reduced niche diversity.

Table 4. Alpha diversity indices.

Sample_Name	chao1	dominance	goods_coverage	observed_features	pielou_e	shannon	simpson
Control1	2506.795	0.002	0.999	2492	0.915	10.322	0.998
MF1	2515.882	0.002	0.999	2502	0.904	10.208	0.998
VC1	2864.731	0.002	0.998	2852	0.898	10.307	0.998
MF1.VC1	2406.176	0.002	0.999	2393	0.899	10.095	0.998
Bch1	2545.500	0.001	0.999	2529	0.920	10.401	0.999
Control2	2682.059	0.001	0.998	2663	0.918	10.443	0.999
MF2	2452.199	0.002	0.999	2442	0.915	10.292	0.998
VC2	2868.429	0.001	0.998	2853	0.921	10.572	0.999
MF2.VC2	2379.850	0.002	0.999	2369	0.908	10.175	0.998
Bch2	2315.000	0.002	1.000	2315	0.917	10.246	0.998

Dominance Index values are uniformly low (~0.001–0.002), indicating minimal dominance by individual species and suggesting strong community balance. Goods Coverage is consistently 0.998–1.000, indicating excellent sequencing depth and comprehensive sample coverage. VC1 and VC2 show the highest species richness, indicating diverse and robust microbial ecosystems. Control2 and Bch1 show balanced microbial evenness, reflecting stable environments. MF1.VC1 and MF2.VC2 exhibit slightly reduced diversity, which could indicate environmental stress or competition-driven dynamics. Goods Coverage near 1.000 across all samples confirms high sequencing depth and completeness.

Rank abundance curve analysis

Samples with longer curves extending further along the X-axis (VC1 and VC2) have greater species richness (Fig. 7). MF1.VC1 and MF2.VC2 have slightly shorter curves, suggesting lower richness. VC1 and VC2 show extended species richness and slightly higher abundance in rare taxa. MF1.VC1 and MF2.VC2 display a sharper drop in abundance, suggesting fewer low-abundance species. MF1.VC1 and MF2.VC2 may indicate conditions favouring dominant species or environmental stress reducing rare taxa survival. Control1, Control2, and Bch1 appear to have balanced distributions with a healthy presence of both abundant and rare taxa. VC1 and VC2 show the highest species richness, indicating a complex and diverse microbial community. MF1.VC1 and MF2.VC2 have lower richness and fewer rare species, suggesting environmental pressures or selective conditions. Control1, Control2, and Bch1 maintain balanced richness and evenness.

Beta diversity analysis

Beta diversity compares microbial community composition across samples (Fig. 8). Taxonomy annotation and sequence abundance were combined into a species abundance table, and phylogenetic relationships were used to calculate unweighted UniFrac distances (Lozupone et al. 2005; Lozupone and Knight 2011). The pairwise beta diversity was evaluated based on Bray-Curtis dissimilarity and UniFrac distance metrics, visualized in a triangular heatmap (Fig. 8). The highest beta diversity values were observed in comparisons involving

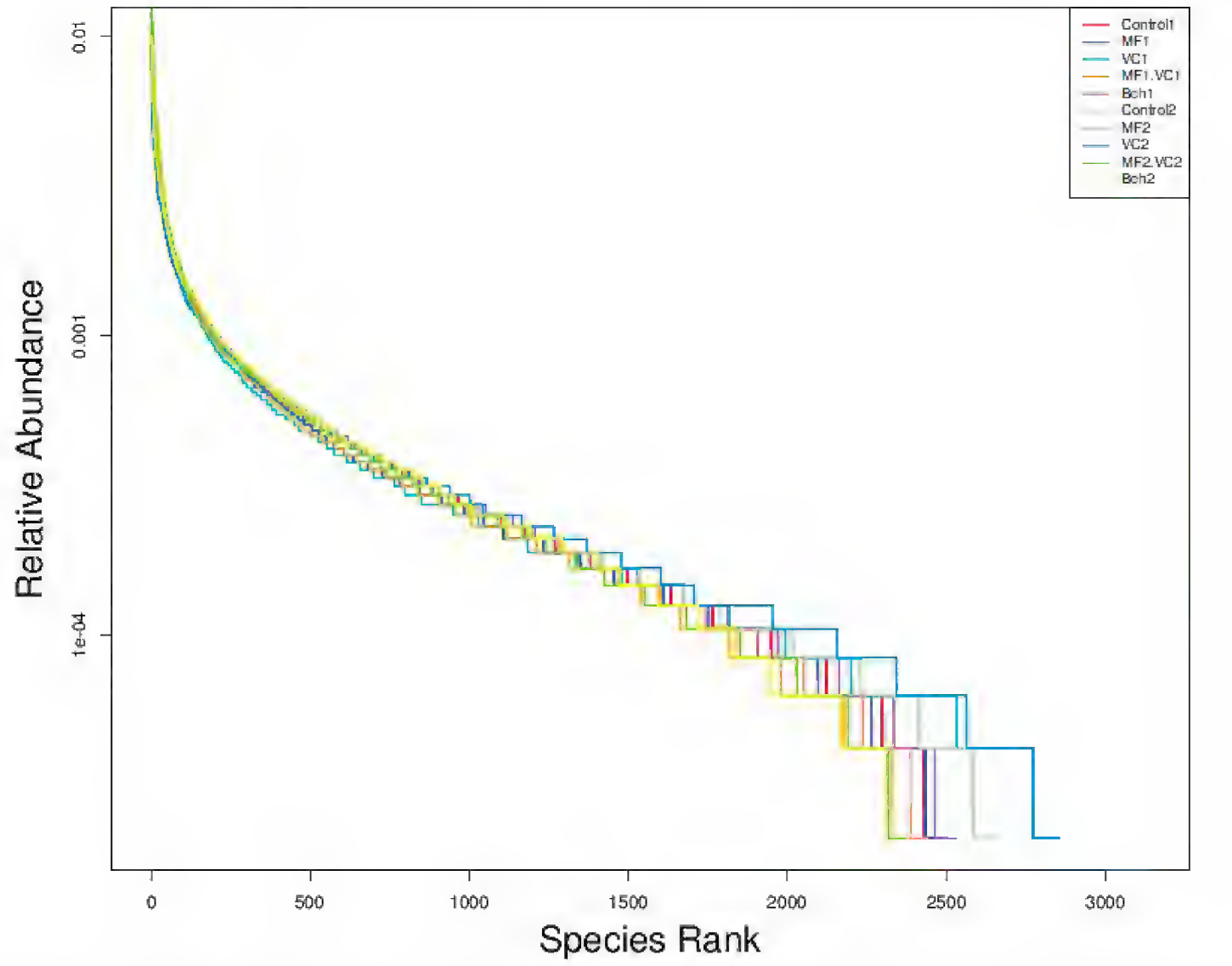

Figure 7. A Rank Abundance Curve visualizes the relative abundance of species across different samples by plotting: X-axis: Species Rank (sorted from most to least abundant) and Y-axis: Relative Abundance (log scale for better visualization of low-abundance species).

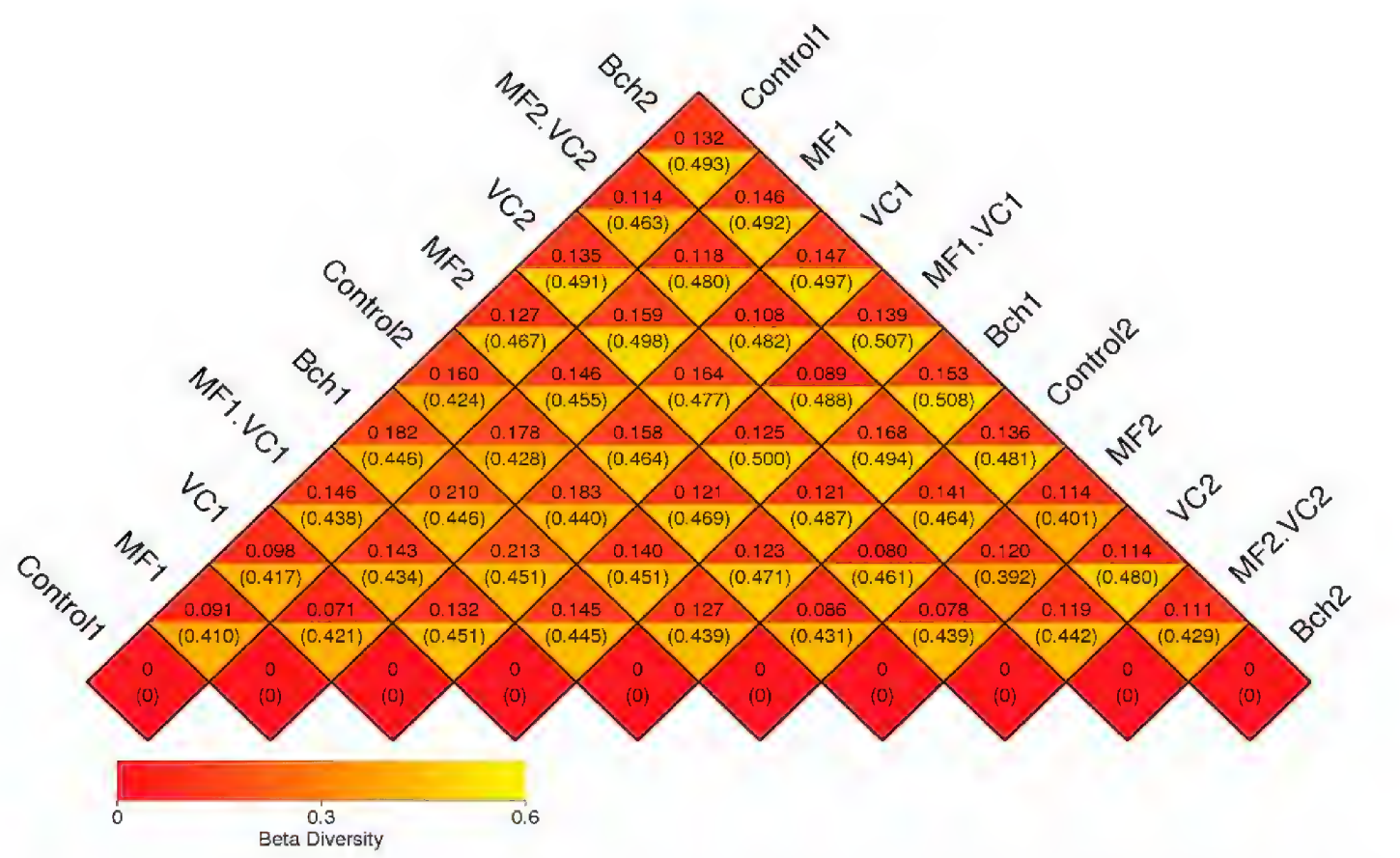

Figure 8. Heatmap of beta diversity distances between samples. Higher values (≈0.6) indicate greater dissimilarity, while lower values (≈0) indicate more similar communities. The color gradient ranges from red (low dissimilarity) to yellow (high dissimilarity).

Bch2, particularly with Control1 (Bray-Curtis = 0.132; UniFrac = 0.493), MF1.VC1 (0.153; 0.508), and MF1 (0.114; 0.463). These elevated values indicate that the biochar treatment Bch2 induced a pronounced shift in community composition, both taxonomically and phylogenetically. Similarly, the combined microbial and vermicompost treatment MF2.VC2 also displayed high dissimilarity

relative to Control1 (0.132; 0.451) and MF1 (0.114; 0.451), suggesting that this treatment alters microbial communities substantially. In contrast, the lowest dissimilarity was observed between Control1 and MF1 (0.098; 0.417), followed by MF1 and VC1 (0.143; 0.434), implying that these individual soil treatments showed limited effects on overall microbial composition, maintaining similarity with the control group. The combination MF1.VC1 resulted in a moderate shift from Control1 (0.182; 0.446), highlighting a possible interaction effect. While taxonomically closer to the control than Bch2, it still demonstrated moderate to high dissimilarity when compared with other treatments such as MF2 (0.146; 0.455) and MF1.VC1 (0.160; 0.424). This suggests that the specific *Bacillus* strain or formulation used in Bch1 induced a unique microbial response. Control1 and Control2 (0.132; 0.451) exhibited measurable dissimilarity due to micro-environmental variability or heterogeneity in microbial baseline conditions. Beta diversity matrix confirms that treatments containing biochar inoculants and MF2.VC2 drive the greatest divergence in microbial community structure, while MF1, VC1, and their individual applications show minimal deviation from the control. These findings are consistent with PCA and PCoA results, reinforcing the conclusion that combined biological amendments can reshape rhizosphere microbial communities, likely through synergistic nutrient effects, microbial interactions, and host plant responses. These results align with previous studies reporting that integrated organic and microbial inputs can significantly modify soil microbial assemblages and enhance functional diversity (Hartmann et al. 2015; Singh et al. 2020). Biochar amendments have been shown to enhance microbial diversity and alter community assembly by providing additional habitat and influencing soil chemistry (Lehmann et al. 2011). Vermicompost applications are consistently associated with improved soil structure and increased abundance of beneficial fungal taxa (Domínguez and Edwards 2011; Pathma and Sakthivel 2012). Likewise, compost inputs promote stable microbial networks and stimulate functional guilds involved in nutrient cycling (Bonanomi et al. 2016). Our results are in line with these studies, suggesting that the combined use of organic inputs and green manuring can foster resilient microbial communities that support both soil health and crop productivity.

Discussion

This study demonstrates that both fertilisation strategy and cropping system significantly shape the structure and functional potential of rhizosphere microbial communities in barley agroecosystems. Using 16S rRNA gene amplicon sequencing, we characterised the microbial taxonomic composition and metabolic capabilities across soils amended with mineral fertiliser, compost, compost-mineral blends, and biochar, under conventional monoculture and oat-vetch intercropping systems. Actinobacteriota and Proteobacteria dominated across all treatments, highlighting their central roles in soil ecosystem functioning (Hartmann et al. 2015; Fierer 2017) (Figs 1–4). The high abundance of Actinomycetota—particularly genera such as *Nocardioides*, *Solirubrobacter*, and *Rubrobacter* suggests a stable and resilient microbial core capable of organic matter decomposition and antibiotic production, aligning with observations in organically managed soils (Lehmann et al. 2011; Zhang et al. 2023) (Fig. 4). Biochar-treated soils displayed

the greatest community divergence, likely due to biochar's capacity to modify soil structure, pH, and moisture retention, fostering unique microbial niches. The MF.VC and Bch treatments enriched a broader range of taxa, including both dominant phyla (e.g., Actinobacteriota, Bacteroidota) and less abundant genera with key functional roles in carbon and nitrogen cycling.

Despite the presence of a shared core microbiome across treatments, microbial richness and evenness were significantly enhanced in organic and green manure-amended plots compared to mineral fertilised and control treatments (Fig. 5). This suggests that integrated nutrient management enhances ecological stability and functional redundancy, which are crucial for resilient soil systems. The phylogenetic and compositional differences observed further support the hypothesis that organic inputs introduce a greater variety of substrates and microhabitats, driving microbial diversification. The abundance of copiotrophic and functionally versatile genera, particularly in green manure plots, confirms the ecological benefit of these practices for sustaining soil biological health and crop productivity. The integration of leguminous green manure (oat-vetch) notably increased alpha diversity, particularly in intercropped and compost-treated plots, supporting previous evidence that legumes stimulate rhizosphere microbial richness through nitrogen fixation and exudate-mediated recruitment (Peoples et al. 2009; Shakya et al. 2013; Philippot et al. 2013; Brooker et al. 2015; Petkova et al. 2025)

The elevated Shannon and Chao1 indices in VC1 and VC2 plots suggest that green manure improves niche availability and substrate heterogeneity, fostering more diverse and even microbial assemblages (Lazcano et al. 2013) (Table 4). The detection of a core microbiome across treatments—comprising over 560 shared species—suggests that barley maintains a stable microbial consortium irrespective of soil amendment. The presence of over 1,000 unique species in VC1 highlights the significant ecological impact of intercropping and green manure incorporation. This enrichment may reflect microbial adaptation to elevated nutrient availability and complex root architectures characteristic of mixed legume-cereal systems (Congreves and Van Eerd 2015; Finney et al. 2016). Importantly, while mineral fertilisers supported high microbial abundance, their sole application was associated with lower evenness and diversity compared with organic or mixed treatments, echoing concerns over long-term microbial homogenization under intensive input regimes (Chen et al. 2018b). Current results suggest that mineral inputs, when used in isolation, may favour copiotrophic taxa and reduce functional redundancy. Together, these findings underscore the synergistic benefits of intercropping and organic fertilisation for maintaining rich, diverse, and functionally robust microbial communities. By integrating oat-vetch green manure with compost-based amendments, farmers can foster a rhizosphere microbiome conducive to nutrient cycling, plant health, and agroecosystem sustainability.

Beta diversity analysis in Fig. 8 revealed distinct community shifts driven by fertilisation type. Bch1 and Bch2 exhibited the most pronounced divergence from other treatments, consistent with biochar's known capacity to alter soil physicochemical properties and provide microhabitats for specialised taxa (Lehmann et al. 2011; Liu et al. 2010).

The clustering trees based on Unweighted and Weighted UniFrac distances reveal distinct patterns in bacterial community structure across treatments

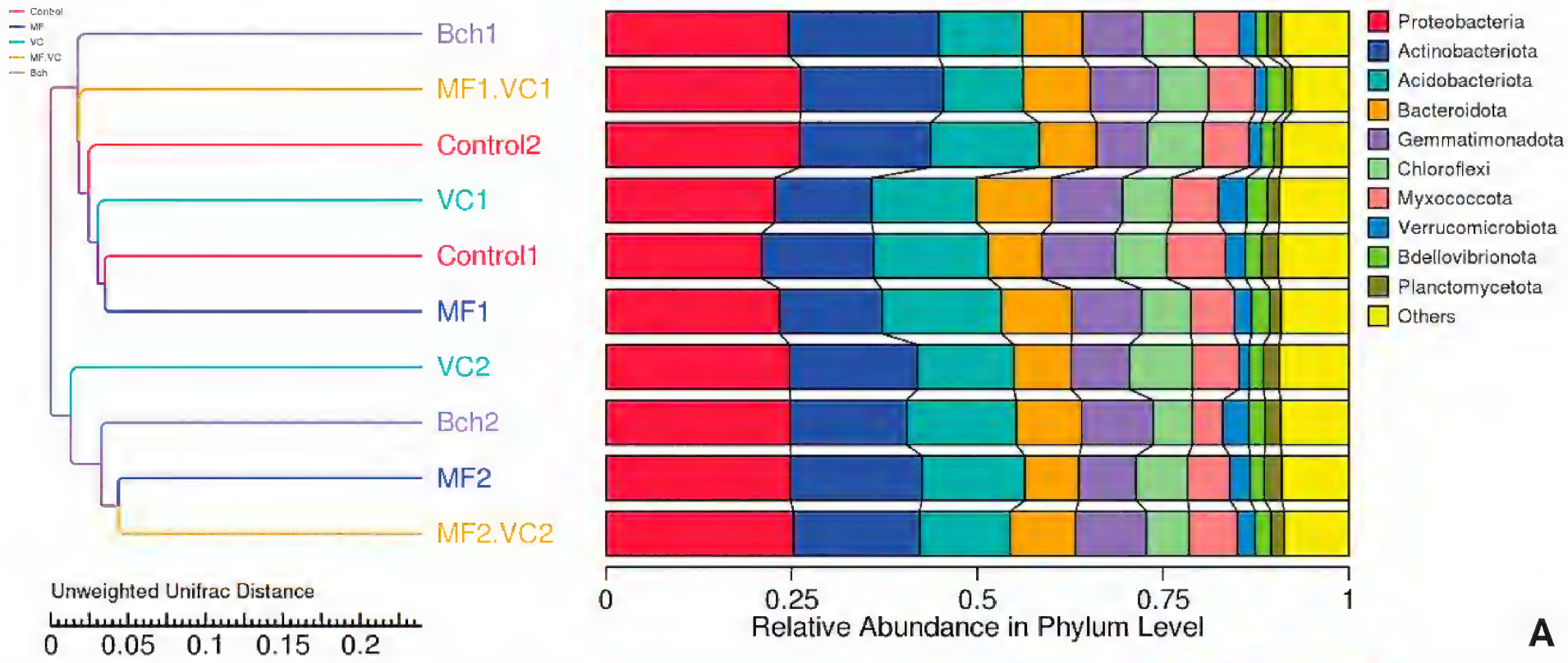

Figure 9. Clustering tree based on Bray-Curtis distance in species abundance (**A**) unweighted and (**B**) weighted Unifrac distance. The dendrogram (left) coupled with a stacked bar plot of relative species abundance (right), offering insight into community composition and similarity among treatments.

(Fig. 9). Unweighted UniFrac (Fig. 9A) considers only the presence or absence of taxa and shows clear separation between treatment groups, especially between Control, MF, and MF.VC samples. This indicates that treatments influence the taxonomic composition, even among low-abundance taxa (Lozupone et al. 2005). In contrast, the Weighted UniFrac tree (Fig. 9B), which accounts for taxon abundances, highlights differences in dominant microbial lineages. MF1.VC1 and MF2.VC2 clusters closely, suggesting a shared enrichment of abundant phyla such as Proteobacteria, Actinobacteriota, and Acidobacteriota, as seen in the relative abundance bar plots (Zhang et al. 2023). The distinct clustering of Bch1 and Bch2 in both trees supports the idea that this treatment promotes a unique microbial assemblage, potentially driven by specific soil or plant-associated factors. These results emphasise that both community composition and abundance profiles are affected by management practices, and combining Unweighted and Weighted UniFrac analyses provides a more comprehensive view of microbial community dynamics (Lozupone and Knight 2011).

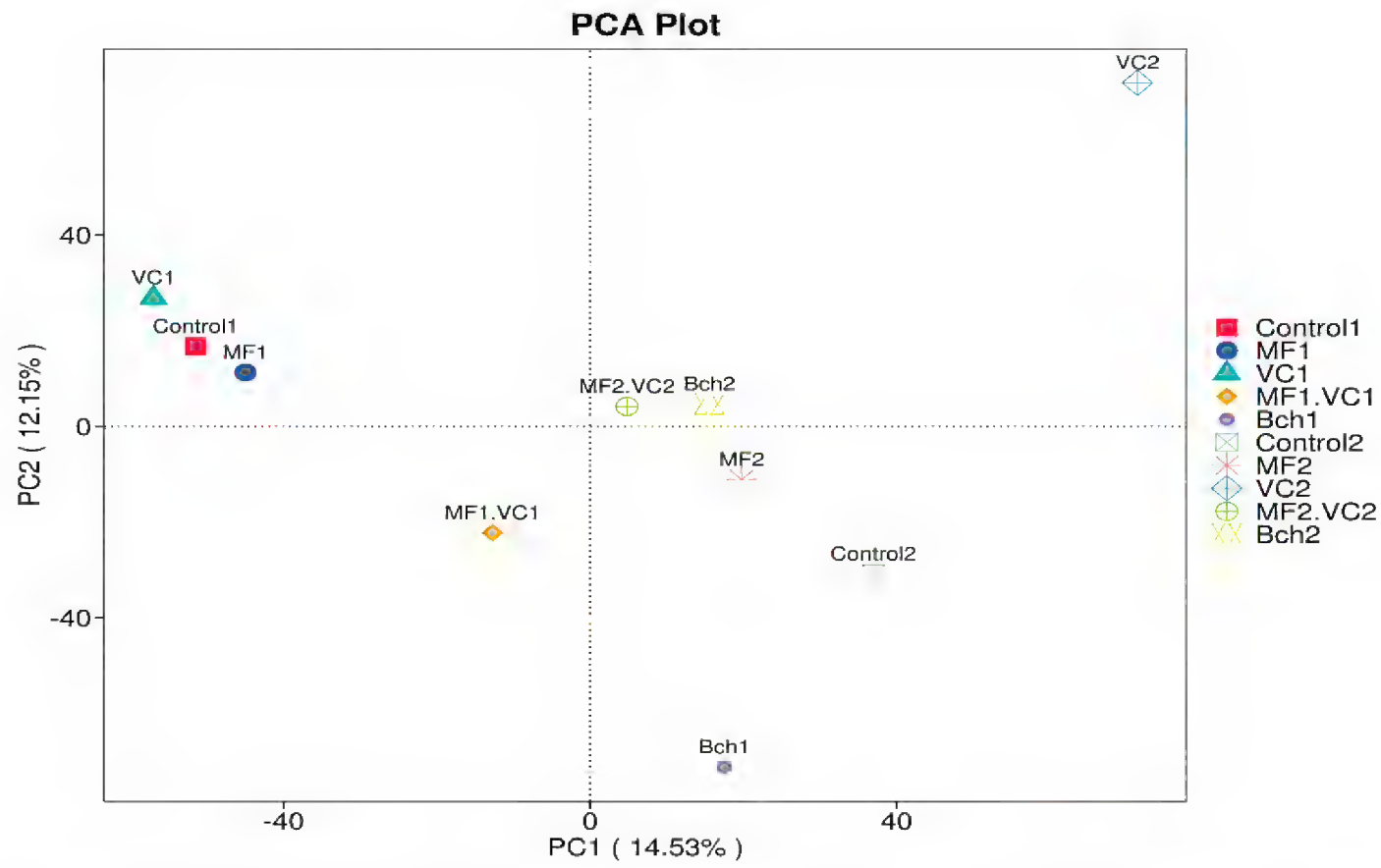

Figure 10. Principal Component Analysis (PCA) of Soil Microbial Communities under Different Fertilisation Treatments.

The PCA plot in Fig. 10 provides a two-dimensional visualization of the variation in community structure among the treatment groups, based on principal component analysis. The first principal component (PC1) accounts for 14.53% of the total variance, while the second principal component (PC2) explains 12.15%, together capturing 26.68% of the observed variation. Distinct clustering patterns were observed among the treatments. Samples from Control1, MF1, and VC1 clustered closely in the upper left quadrant, indicating that these groups shared similar microbial or biochemical profiles and responded similarly to the tested conditions. In contrast, Control2 and MF2 were located in the lower right quadrant, clearly separated from Control1 and MF1, suggesting divergent profiles potentially associated with the effects of the treatments or environmental differences between replicates. MF2.VC2 and Bch2, grouped near the center of the plot, implying moderate or mixed responses that do not strongly drive variation along either PC1 or PC2. VC2 appeared as a clear outlier in the top right quadrant, reflecting a pronounced deviation in community structure, especially along PC2. Similarly, Bch1 was located distantly from other groups in the lower quadrant, suggesting a unique treatment effect or strain-specific response. MF1.VC1, positioned between the clusters of MF1 and VC1, displayed an intermediate profile, indicative of a partial interaction effect. Likewise, the combined treatment MF2.VC2 demonstrated a distinct but centrally located profile, suggesting a complex or balanced interaction between the individual components. PCA highlights the treatment-driven differentiation of the microbial or biochemical community structure. The close association of certain treatments (Control1, MF1, and VC1) and the divergence of others (MF2, VC2, Bch1) underscore the varied effects of experimental conditions. Bch2 also suggests potential intra-group variability or sensitivity to environmental or procedural factors. These findings emphasize the relevance of treatment-specific and interaction-dependent influences on the underlying biological system.

Principal Coordinates Analysis (PCoA) in Fig. 11 was performed to assess beta-diversity and visualize differences in microbial community composition

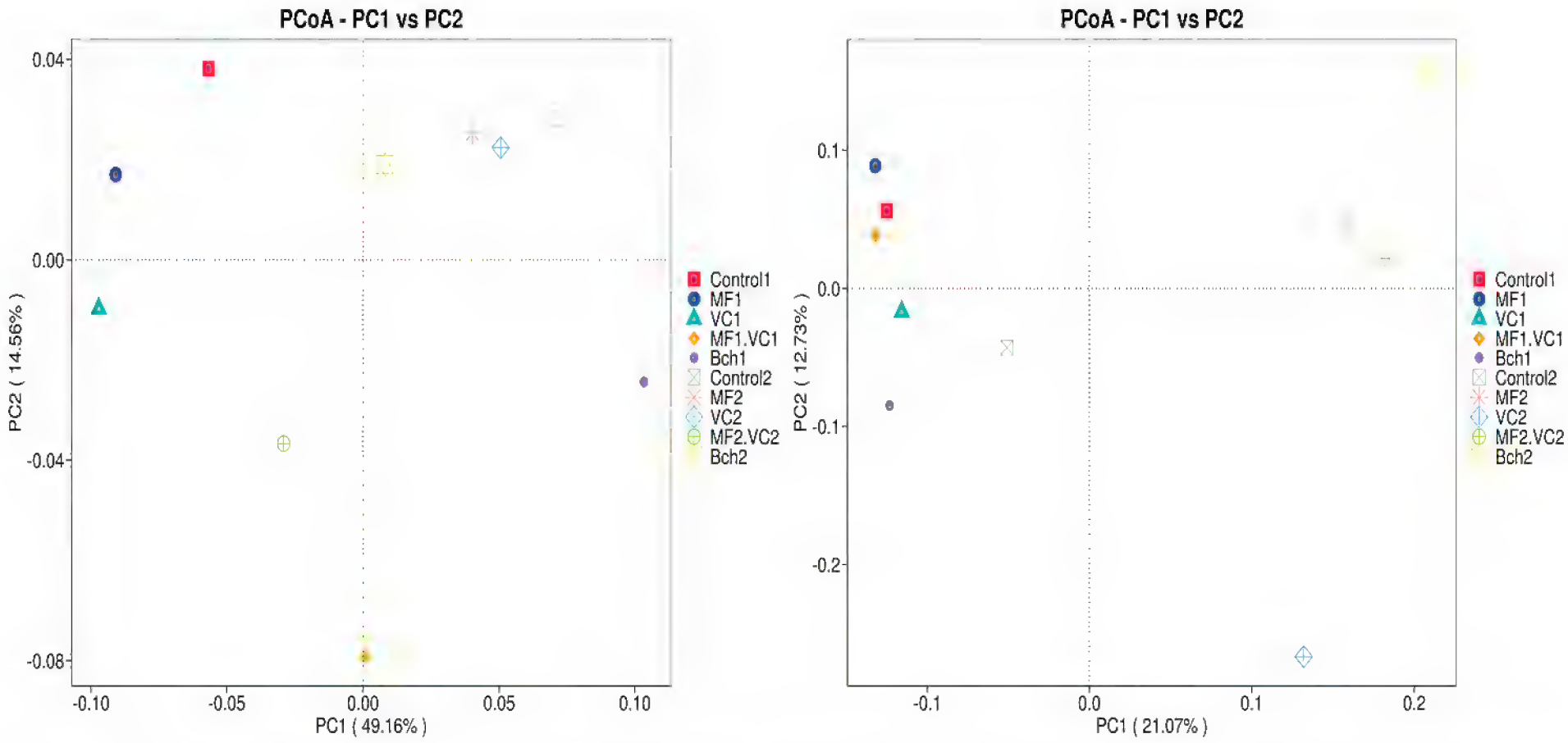

Figure 11. Principal Coordinates Analysis (PCoA) of Soil Microbial Communities Based on UniFrac Distances.

across the experimental treatments. This method allows for dimensionality reduction based on pairwise dissimilarity metrics and is commonly used to evaluate compositional shifts in microbial communities (Lozupone et al. 2005). In the left panel of Fig. 11, the first two principal coordinates explain 49.16% and 14.56% of the variance, respectively. A clear clustering of MF1, VC1, and Control1 is observed in the upper left quadrant, suggesting compositional similarity under these treatments. In contrast, MF2.VC2 and Bch2 are distinctly separated in the right half of the plot, reflecting a marked divergence in microbial community structure. Bch1 and MF1.VC1 show isolated positions, indicating unique microbial signatures that differ from both control and other treatment groups.

The right panel of the PCoA plot, based on a different distance metric, reveals similar but more dispersed clustering. Here, PC1 and PC2 explain 21.07% and 12.73% of the total variation, respectively. Bch2 and MF2.VC2 cluster tightly in the upper right quadrant, indicating a strong treatment effect from the combined application of Bacillus-based biostimulants and vermicompost. VC2 appears as an outlier in the lower right quadrant, confirming a distinct microbial community composition. Meanwhile, MF1, Control1, and MF1.VC1 cluster in the upper left quadrant, reinforcing their compositional similarity. PCoA results confirm that specific treatments—particularly MF2, VC2, and Bch-based amendments—induce significant shifts in microbial community structure compared to the control. The clustering patterns indicate that combinations of microbial fertilizers and organic inputs can lead to synergistic or additive effects, reshaping the rhizosphere microbiome. These findings are consistent with previous studies showing that organic and microbial amendments drive distinct microbial assemblages and influence soil ecological function (Hartmann et al. 2015; Ai et al. 2015; Singh et al. 2020).

The influence of various fertilisation strategies on soil microbial communities was assessed under two contrasting cropping systems: a conventional system with direct barley sowing (no prior cover crop), and an intercropping system utilising oat-vetch green manure before barley cultivation. In the absence of fertilisation (Control), microbial communities remained stable under

conventional management, dominated by Actinomycetota. The intercropped control plots supported slightly higher microbial richness and functional diversity, consistent with findings that legume-based green manures enhance soil microbiota even without nutrient amendments (Zhang et al. 2018; Zhou et al. 2021). Mineral fertilisation induced a selective increase in stress-tolerant taxa such as Nocardioides and Rubrobacteraceae, leading to reduced diversity in conventionally managed soils. In contrast, the green manure intercropping system preserved a more balanced microbial structure and facilitated the appearance of Acidobacteriota and Chloroflexota, phyla known for their roles in organic matter turnover and environmental resilience (Janssen 2006; Kielak et al. 2016). Compost application enriched decomposer bacteria under conventional barley, especially Nocardioides, but had a stronger effect under intercropping by promoting Solirubrobacteraceae and less abundant phyla. The inclusion of compost significantly enhanced microbial diversity and functionality, particularly when combined with green manure practices, aligning with previous reports that organic amendments stimulate microbial richness and promote beneficial taxa (Lazcano et al. 2013; Povilaitis et al. 2020).

The combination of compost and mineral fertilisation led to strong dominance by *Nocardioides* and Frankiales under conventional management. Under intercropping, this combination promoted greater microbial evenness and enriched rare taxa such as Gaiella occulta. This suggests a synergistic effect between organic and inorganic inputs when used with green manure, enhancing microbial functional redundancy—an important indicator of soil ecosystem resilience (Banerjee 2021). Biochar application supported distinct microbial assemblages dominated by carbon-degrading bacteria under conventional barley cultivation. The intercropping-biochar combination resulted in the broadest microbial spectrum, including enrichment of niche-specific and rare taxa as *Gaiella*. These results corroborate the hypothesis that biochar in combination with legume-based green manures enhances microbial niche complexity and supports specialised functional groups (Quilliam et al. 2013; Lehmann et al. 2011).

The oat-vetch green manure system improved microbial richness, evenness, and functional diversity across all fertilisation treatments. Intercropping not only mitigated the potential negative effects of mineral fertilisation but also enhanced the performance of organic amendments. These findings support the integration of green manures with compost or biochar as effective strategies to promote diverse and ecologically robust soil microbiomes in barley-based cropping systems. From an ecological standpoint, the application of legume-based green manure fostered microbial resilience and ecosystem complexity, supporting a broader range of taxa and potentially increasing functional redundancy. Functionally, these treatments are likely to enhance nutrient cycling, organic matter turnover, and plant-microbe interactions, which are critical for long-term soil fertility and crop productivity. These findings demonstrate that integrated nutrient management combining green manure with organic amendments is a viable strategy to promote diverse, ecologically stable, and functionally robust microbial communities in cereal cropping systems. Such practices are key to advancing soil health, sustainability, and agroecological resilience under modern agricultural pressures. While the present research concentrates on bacterial communities, we acknowledge that fungi and protozoa interact closely with bacteria in driving decomposition and nutrient mineralisation. Future studies

integrating bacterial, fungal, and protozoan analyses will provide a more comprehensive view of soil functional ecology under these agroecological practices.

Conclusions

Growing intercropped oat-vetch and incorporating them at the ripening stage as green manure, and applying vermicompost, fosters more diverse, functionally versatile, and ecologically resilient rhizosphere microbiomes compared to soils without green manure or mineral inputs.. The bacterial biome represents only a part of the complex soil microbiome, and that future metagenomic work should encompass fungal and protozoan contributions to fully elucidate soil ecosystem functioning. These findings advocate for the adoption of intercropping with legume green manure and tailored organic amendments to support sustainable cereal production. Ecological and functional benefits of the current study are the improved diversity under green manure and combined treatment, which likely translates into more robust nitrogen and carbon turnover, driven by a broader suite of degradative and biosynthetic pathways. Communities in vermicompost and combined fertilisation plots exhibited greater phylogenetic dispersion, suggesting improved capacity to withstand environmental perturbations. Biochar's ability to stabilise soil pH and moisture appears to foster specialised consortia, potentially improving long-term soil structure and plant-microbe interactions.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

Conceptualization, S.S.; methodology, S.S. and M.P.; formal analysis, M.P., V.P., N.M. and I.N.; investigation, M.P., V.P., I.N. and S.S.; resources, S.S.; writing – original draft preparation, M.P. and S.S.; writing – review and editing, M.P., S.S., I.R.; supervision, S.S.; funding acquisition, S.S. All authors have read and agreed to the published version of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text.

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